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From: STIC-EIC1600/2900
Sent: Monday, August 18, 2008 6:54 AM
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From: Salimi, Ali
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Please, provide the following. Thank you!

1)Wang et al, Virology, 1992, Vol. 223, pp. 41-50.
2) Lu et al, Virology, 1995, Vol. 214, pp. 222-228.

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The RNA-Binding and Effector Domains of the Viral NS1 Protein Are Conserved to Different Extents among Influenza A and B Viruses

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The NS1 protein of the influenza A/Udorn/72 virus possesses two important functional domains: an RNA-binding domain near the amino-terminal end and an effector domain in the carboxyl half of the molecule. Though the NS1 proteins of influenza A and B viruses share little sequence homology, an RNA-binding domain with the same activities is preserved in the NS1 protein of influenza B/Lee/40 virus. The RNA-binding domains of the NS1 proteins of these influenza A and B viruses share the following properties: (i) they specifically bind to the same three RNA targets, poly(A), U6 snRNA, and double-stranded (ds) RNA; (ii) a polypeptide containing an amino-terminal sequence of the protein possesses all the RNA-binding activity of the full-length protein and exists in the form of a dimer; (iii) the binding to U6 snRNA causes an inhibition of pre-mRNA splicing *in vitro*; and (iv) the binding to dsRNA blocks the activation of the PKR kinase *in vitro*. The conservation of the RNA-binding domain of the NS1 protein among influenza A and B viruses strongly suggests that this domain is required for the replication of all these influenza viruses. In contrast, the NS1 protein of influenza B virus (NS1B protein) lacks an effector domain that functions like that of the NS1 protein of influenza A virus (NS1A protein). The effector domain of the NS1A protein is required for two of its *in vivo* activities: the inhibition of the nuclear export of poly(A)-containing mRNA and the inhibition of pre-mRNA splicing. The NS1B protein lacks these two *in vivo* activities. In addition, a naturally occuring, truncated NS1A protein lacks such an effector domain. Consequently, an effector domain that functions like that of full-length NS1A proteins is not absolutely required for the replication of influenza A and B viruses. We discuss the implications of these results for the roles of the RNA-binding and effector domains of the NS1 protein during infection by influenza A and B viruses.

INTRODUCTION

Influenza viruses have been classified into types A, B, and C based on the immunological relatedness of their nucleocapsid (NP) proteins (Kilbourne, 1975). Thus, the influenza A virus NP protein is immunologically unrelated to the NP proteins of influenza B and C viruses. Of the three types, influenza A viruses have been the most intensively studied, but not all of the properties of A viruses are shared by the B and C influenza viruses (reviewed in Lamb, 1989).

The genomes of influenza A and B viruses are similar in that both are composed of eight segments of single-stranded RNA of negative polarity, and most of the proteins encoded by the corresponding genome segments serve similar functions (Lamb, 1983). However, the sizes of both the genome segments and their encoded proteins differ between the A and B viruses (reviewed in Lamb, 1989). In addition, fundamental differences in the mechanisms of expression of several of the viral genes have been documented, specifically involving the M (matrix) and NA (neuraminidase) genes. Segment 7 of both influenza A and B viruses encode two proteins, but two different posttranscriptional mechanisms are employed to express the two proteins. With influenza A viruses, the colinear transcript of the M genome segment, the M1 mRNA, encodes the M1 (matrix) protein, and the M2 mRNA that encodes the M2 (ion channel) protein is produced by splicing of M1 mRNA in the nucleus (Lamb and Choppin, 1981). This splicing is controlled by both the complex of the three viral polymerase proteins and the cellular SF2/ASF splicing factor (Shih *et al.*, 1995; Shih and Krug, 1996). In contrast, with influenza B viruses, the colinear transcript of the M segment, the M1 mRNA, is not spliced, but rather is translated as a bicistronic mRNA in the cytoplasm into two proteins, M1 (matrix) and a protein called BM2 (Horvath *et al.*, 1990). However, the BM2 protein of influenza B virus has no homology with the influenza A virus M2 ion channel protein. The presumed B virus counterpart to the A virus M2 protein is the NB protein, which is encoded by the genome segment that also encodes the NA protein (Williams and Lamb, 1986). The transcript of the B virus NA segment, NA mRNA, is a bicistronic mRNA (Shaw *et al.*, 1983), whereas the influenza A virus NA mRNA is monocistronic and is translated into only the NA protein (Colman and

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Ward, 1985). Fundamental differences in the biological properties of influenza A and B viruses have also been found. For example, influenza B virus, but not A virus, infections in humans are associated with the development of Reye's syndrome (Douglas, 1975).

Both influenza A and B viruses encode a protein called NS1 (nonstructural protein 1) that is the translation product of the colinear transcript (NS1 mRNA) of the smallest genome RNA segment (Lamb and Lai, 1980; Briedis *et al.*, 1981). The NS1 proteins of the A and B viruses share very little sequence homology, and the NS1B proteins are usually larger than the NS1A proteins (Briedis and Lamb, 1982). For example, the NS1 protein encoded by B/Lee/40 virus is 281 amino acids long, whereas the NS1 protein encoded by A/Udorn/72 is only 237 amino acids long.

It is not known whether the NS1 proteins of the influenza A and B viruses perform similar functions during infection. Several functions of the NS1 protein of influenza A virus have recently been elucidated (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Lu *et al.*, 1994, 1995; Qian *et al.*, 1994; Qiu and Krug, 1994; Qiu *et al.*, 1995). The NS1A protein of A/Udorn/72 virus contains two functional domains, an RNA-binding domain near the amino terminal end and an effector domain in the carboxyl half of the molecule (Qian *et al.*, 1994). The NS1A protein has been shown to function in several posttranscriptional steps. First, this protein binds to the poly(A) of mRNA (Qiu and Krug, 1994) and inhibits the nuclear export of poly(A)-containing mRNAs (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Qian *et al.*, 1994; Qiu and Krug, 1994). In addition, the NS1A protein binds to a stem-bulge in a key spliceosomal RNA, U6 small nuclear RNA (snRNA) (Qiu *et al.*, 1995), and inhibits pre-mRNA splicing both *in vivo* (Fortes *et al.*, 1994; Lu *et al.*, 1994) and *in vitro* (Lu *et al.*, 1994; Qiu *et al.*, 1995). Only the RNA-binding domain is needed to inhibit splicing *in vitro*, but both the RNA-binding and effector domains are required for the inhibition of poly(A)-containing mRNA nuclear export and of pre-mRNA splicing *in vivo* (Lu *et al.*, 1994; Qian *et al.*, 1994). Finally, the NS1A protein binds to double-stranded RNA (dsRNA) in a sequence-independent manner (Hatada and Futada, 1992; Lu *et al.*, 1995), thereby blocking the dsRNA-mediated activation of the cellular PKR kinase *in vitro* (Lu *et al.*, 1995). If activated, the PKR kinase phosphorylates the α subunit of the translation initiation factor eIF2, causing an inhibition of cellular and viral protein synthesis (reviewed in Rhoads, 1993; Samuel, 1993). Only the RNA-binding domain of the NS1A protein is needed for blocking PKR kinase activation *in vitro* (Lu *et al.*, 1995).

The present study demonstrates that the NS1 protein of influenza B virus shares some, but not all, of the properties of the NS1 protein of influenza A virus. Like its A virus counterpart, the NS1 protein of influenza B virus is an RNA-binding protein and in fact binds to the same three RNA targets as the NS1A protein. The binding of this protein to U6 snRNA and dsRNA results in the inhibition of pre-mRNA splicing *in vitro* and in the blocking of the activation of the PKR kinase, respectively, as previously documented for the NS1A protein (Lu *et al.*, 1994, 1995; Qiu *et al.*, 1995). However, the NS1 protein of influenza B virus lacks two of the *in vivo* activities exhibited by the NS1 protein of influenza A virus. Thus, neither the inhibition of the nuclear export of mRNA nor the inhibition of pre-mRNA splicing occurs in cells transfected with the NS1B gene. This strongly suggests that the NS1B protein lacks an effector domain that functions like that of the NS1 protein of most influenza A virus strains. There is one influenza A virus strain, however, which encodes a NS1 protein that lacks the carboxyl region including the effector domain (Norton *et al.*, 1987), and we show that such a naturally occurring truncated NS1A protein behaves similarly to the full-length NS1B protein in all of our *in vitro* and *in vivo* functional assays.

MATERIALS AND METHODS

Plasmid construction and protein purification

All the NS1 genes described in this paper contain a 3′ splice site mutation to eliminate the production of NS2 mRNA (Lamb, 1989). The NS1 gene of influenza A/Udorn/72 virus has been described previously (Alonso-Caplen *et al.*, 1992; Qiu and Krug, 1994). The NS1 gene of influenza B/Lee/40 virus in the pBR322 plasmid was provided by Robert A. Lamb (Briedis and Lamb, 1982). PCR was used to introduce *Hin*dIII and *Sma*I sites at the 5′ and 3′ ends, respectively, of the DNA sequence encoding the NS1B protein. This NS1B sequence was cloned into the *Hin*dIII and *Sma*I sites of the pBC12 plasmid, and the resulting plasmid was used for transfections. The same NS1B sequence was blunt-ended and ligated into the unique *Sma*I site of the pGEX-3X (Phamacia) expression vector. The resulting vector was transformed into *Escherichia coli* JM101 to express the GST–NS1B fusion protein. To prepare GST fusion proteins containing the 68, 78, 93, or 103 amino-terminal amino acids of the NS1B protein, two stop codons (TAGTAA) were introduced into the appropriate positions of the NS1B reading frame using PCR. The NS1 gene of influenza A/Turkey/Oregon/71 virus in a pBR322 plasmid was provided by Peter Palese (Norton *et al.*, 1987). The appropriate 10 nucleotides were inserted into this NS1 gene using PCR to generate the NS1 gene of influenza A/Duck/Alberta/60/76 virus (Norton *et al.*, 1987). The GST fusion proteins were purified and, where indicated, were cleaved using factor Xa as described previously (Qiu and Krug, 1994). The purity of all proteins were established by gel electrophoresis followed by Coomassie blue staining.

RNA-binding assays

The following ^{32}P-labeled RNA targets were prepared as previously described: U6 snRNA (Qiu *et al.*, 1995); pGEM1 dsRNA (Lu *et al.*, 1995); poly(A) (Qiu and Krug, 1994). The RNA-binding assays were carried out as described previously (Qiu and Krug, 1994; Lu *et al.*, 1995). Briefly, the indicated amounts of protein and target RNA were incubated on ice for 30 min in 20-μl binding reactions containing 43 m*M* Tris–HCl, pH 8.0, 50 m*M* KCl, 8% glycerol, 2.5 m*M* dithiothreitol, 50 μg/ml *E. coli* tRNA, and 0.5 units/μl RNasin. All the proteins used for binding were cleaved from the GST fusion proteins using factor Xa. The protein–RNA complexes were resolved from free RNA by nondenaturing polyacrylamide gel electrophoresis at 4°. For the U6 snRNA and dsRNA binding assays, 6 and 10% gels were used, respectively. The gels were run at 150 V for 3 hr using 0.045 *M* Tris–borate, 0.001 *M* EDTA as running buffer. For poly(A) binding assays, a 4% gel and a running buffer of 43 m*M* Tris–HCl, pH 8.0, 50 m*M* KCl were used. The gels were run at a constant current of 20 mA for 16 hr with buffer recirculation.

Chemical modification/interference analysis

Chemical modification/interference analysis was carried out as described previously (Qiu *et al.*, 1995), except that the U6 snRNA substrate (3′-^{32}P-labeled) was treated with anhydrous 3 *M* NaCl/hydrazine (for C ≫ U modification) or with 50% hydrazine/50% H_2O (for U modification) as described by Peattie (1979).

In vitro translation

Nuclease-treated rabbit reticulocyte extracts were programmed with luciferase mRNA using the TNT coupled Transcription/Translation system (Promega). The reaction mixtures contained [^{35}S]methionine, an amino acid mixture (minus methionine), Sp6 RNA polymerase, and DNA encoding luciferase mRNA in a final volume of 10 μl, as previously described (Lu *et al.*, 1995).

Glutathione Sepharose bead affinity selection

A purified fusion protein, GST fused to a NS1B sequence (2 μg), was combined with 5 μl of *in vitro* translated ^{35}S-labeled, full-length NS1B protein, 20 μl glutathione Sepharose 4B beads (Pharmacia), and 0.4 ml Ipp150 buffer (10 m*M* Tris–HCl, pH 8.0, 0.15 m*M* NaCl, and 0.1% Nonidet P-40) in a 1.5-ml Eppendorf tube. The tubes were rotated at 4° for 90 min. After the beads were washed extensively, the labeled bound protein was eluted as described previously (Nemeroff *et al.*, 1995). The resulting eluate was analyzed by electrophoresis on a 14% SDS–PAGE gel.

In vitro splicing assays

To prepare a splicing precursor, a β-globin minigene in pGEM1 linearized by *Bam*HI was transcribed in the presence of [α-^{32}P]GTP by SP6 RNA polymerase, as described previously (Agris *et al.*, 1989). Splicing reactions, in a final volume of 25 μl, were carried out with 10 μl of HeLa cell nuclear extract essentially as described previously (Nemeroff *et al.*, 1992; Lu *et al.*, 1994). The splicing reactions were incubated at 30° for 2 hr. RNA was extracted and analyzed by electrophoresis on a 5% denaturing gel (Lu *et al.*, 1994).

Transfection and S1 analysis

Transfection of 293 cells was carried out as described previously (Alonso-Caplen *et al.*, 1992). The 5′-end-labeled single-stranded probes were prepared, and S1 analysis was carried out as described previously (Alonso-Caplen *et al.*, 1992; Lu *et al.*, 1994; Qian *et al.*, 1994).

RESULTS

The NS1 protein of influenza B virus binds to the same RNA targets as the NS1 protein of influenza A virus

The NS1 protein of influenza A virus (NS1A protein) binds to three RNA targets: U6 snRNA, dsRNA, and poly(A) (Qiu and Krug, 1994; Lu *et al.*, 1995; Qiu *et al.*, 1995). To determine whether the NS1 protein of influenza B virus (NS1B protein) has similar RNA-binding activities, purified NS1B protein (cleaved from a GST–NS1B fusion protein) was incubated with each of these RNA targets, and the formation of RNA–protein complexes was assayed by native gel electrophoresis. Figure 1 shows the results obtained with the U6 snRNA target. Increasing concentrations of the NS1B protein shifted increasing amounts of U6 snRNA into protein–RNA complexes that migrated with lower mobilities than the free U6 snRNA during gel electrophoresis (Fig. 1A). As shown previously (Qiu *et al.*, 1995), the GST protein alone does not form a complex with U6 snRNA (data not shown). The protein–U6 snRNA complexes formed with the higher concentration (400 n*M*) of the GST–NS1B protein had a lower mobility than the complexes formed with lower concentrations of the GST–NS1B protein. The same phenomenon was observed with the NS1A protein (Qiu *et al.*, 1995). The NS1B protein titration curve is similar to that previously obtained with the NS1A protein (Qiu *et al.*, 1995). In fact, a chemical modification/interference experiment showed that the NS1B protein binds to the same stem-bulge structure in U6 snRNA as the NS1A protein (Qiu *et al.*, 1995) (Figs. 1B and 1C.).

The NS1B protein also effectively formed complexes

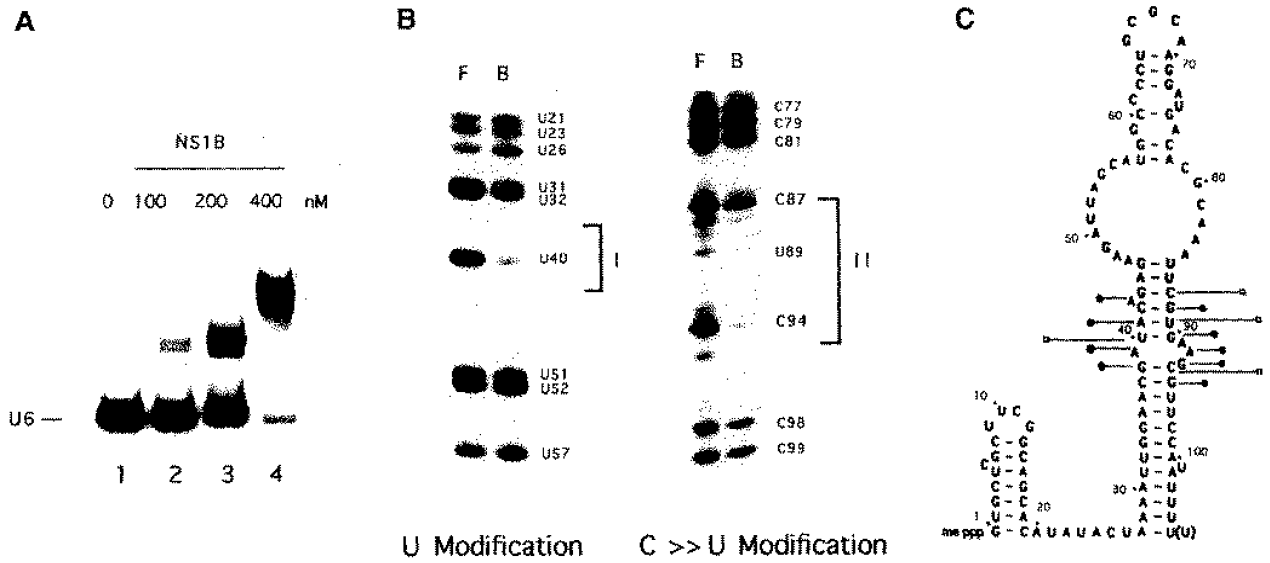

FIG. 1. The NS1B protein binds to the same stem-bulge in U6 snRNA as does the NS1A protein. (A) RNA-binding assay. ^{32}P-labeled U6 snRNA (10,000 cpm, 1 n*M*) was mixed with the indicated concentrations of the NS1B protein under standard RNA-binding conditions as described under Materials and Methods (lanes 2–4). The protein–RNA complexes were separated from free RNA by nondenaturing gel electrophoresis. Lane 1, U6 snRNA alone. (B) Chemical modification/interference assay. U6 snRNA (3′ ^{32}P-labeled) was treated as described under Materials and Methods to modify either its U residues or its C residues (C ≫ U). The modified U6 snRNA was incubated with the NS1B protein, the U6 snRNA–NS1B protein complexes were isolated by nondenaturing gel electrophoresis, and the U6 snRNA was phenol extracted. This constituted the bound, or B, U6 snRNA. The U6 snRNA not bound to the NS1B protein constituted the free, or F, U6 snRNA. The F and B U6 snRNAs were cleaved with aniline, and the resulting products were resolved by denaturing gel electrophoresis. Strong interference was observed in two regions (I and II). (C) Comparison of the U6 snRNA nucleotides protected by the NS1B protein (—□, pyrimidines protected) and by the NS1A protein (—●, purines protected).

with dsRNA (Fig. 2A). The dsRNA used in this experiment was 55 bp long and was generated by annealing the sense and antisense transcripts of the polylinker of the pGEM1 plasmid. Again, the NS1B protein titration curve is similar to that obtained with the NS1A protein (Lu *et al.*, 1995). Finally, the NS1B protein formed complexes with poly(A) (Fig. 2B). Both the NS1B and NS1A proteins were less efficient in binding poly(A) than in binding U6 snRNA or dsRNA (compare lanes 2 and 5 of Fig. 2B to Figs. 1A and 2A). The specificity of the binding to poly(A) was established by a competition experiment. An excess amount of unlabeled poly(A) eliminated complex formation (Fig. 2B, lanes 3 and 6), whereas the same amount of unlabeled poly(C) had no effect on complex formation (lanes 4 and 7). The NS1A and NS1B proteins behaved similarly in this competition experiment.

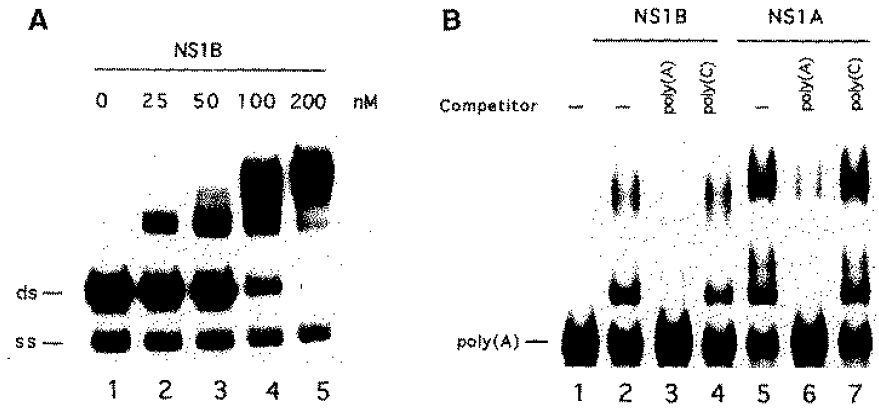

FIG. 2. The NS1B protein, like the NS1A protein, binds to two other RNA targets, dsRNA and poly(A). (A) ^{32}P-labeled pGEM1 dsRNA (10,000 cpm, 1 n*M*) was incubated with increasing concentrations of the NS1B protein (lanes 2–5). Lane 1, pGEM1 dsRNA alone. ds, double-stranded RNA; ss, unannealed single-stranded RNAs. (B) ^{32}P-labeled poly(A) (10,000cpm, 0.25 n*M*) was mixed with 800 n*M* NS1B protein (lanes 2–4) or 800 n*M* NS1A protein (lanes 5–7) in the absence of a competitor RNA (lanes 2 and 5) or in the presence of 1 μg unlabeled poly(A) (lanes 3 and 6) or poly(C) (lanes 4 and 7). Lane 1, poly(A) alone.

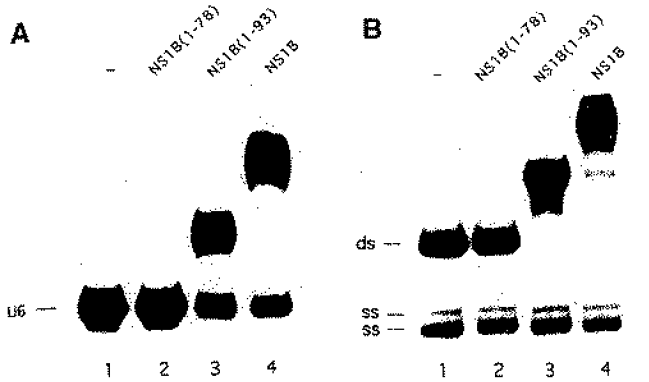

FIG. 3. An amino-terminal polypeptide fragment of the NS1B protein possesses the specific RNA-binding activities of the full-length protein. Purified GST fusion proteins (200 *nM*) containing either the entirety of the NS1B protein (lane 4) or various lengths (78 or 93 amino acids) of the amino-terminus fragment of the NS1B protein (lanes 2 and 3), after cleavage with factor Xa, were mixed with ^{32}P-labeled U6 snRNA (A) or pGEM1 dsRNA (B) under standard RNA-binding conditions. The mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, U6 snRNA (A) or dsRNA (B) alone.

Like its NS1A counterpart, a polypeptide comprising the amino terminus of the NS1B protein possesses the RNA-binding and dimerization activities of the full-length protein

The RNA-binding domain of the NS1A protein is located near its N-terminal end (Qian *et al.*, 1994), and a polypeptide comprising the amino-terminal 73 amino acids possesses all the RNA-binding activities of the full-length protein (Qian *et al.*, 1995). To determine whether an amino-terminal fragment of the NS1B protein also constituted its RNA-binding domain, several amino-terminal fragments of the NS1B protein were tested for their abilities to bind U6 snRNA and dsRNA (Fig. 3). The fragment containing the 78 amino-terminal amino acids of the NS1B protein was inactive in binding either U6 snRNA or dsRNA (lane 2, Figs. 3A and 3B). In contrast, the polypeptide containing the 93 amino-terminal amino acids of the NS1B protein bound both U6 snRNA and dsRNA as efficiently as the full-length protein (compare lanes 3 and 4). Consequently, the 93-amino-acid, amino-terminal polypeptide of the NS1B protein (NS1B (1–93)) has the same RNA-binding activities as the 73-amino-acid, amino-terminal polypeptide of the NS1A protein (NS1A (1–73)) (Qian *et al.*, 1995). The binding abilities of two other amino-terminal fragments of the NS1B protein were also determined: NS1B (1–103) was fully active, whereas NS1B (1–68) had no detectable binding ability (data not shown).

The full-length NS1 protein of influenza A virus exists as a dimer *in vitro* both in the absence of its RNA target and when it is bound to U6 snRNA. Mutational analysis indicated that the RNA-binding and dimerization domains of the protein are coincident, strongly suggesting that the NS1A protein binds to its RNA targets in the form of a dimer (Nemeroff *et al.*, 1995). The NS1A (1–73) polypeptide retains the dimerization activity of the full-length protein (Qian *et al.*, 1995). To determine whether the NS1 protein of influenza B virus also interacted with itself via its RNA-binding domain, we carried out the glutathione selection assay depicted in Fig. 4A. GST fusion proteins containing various lengths of the amino terminus of the NS1B protein were bound to glutathione Sepharose beads, and the ability of an ^{35}S-labeled full-length, nonfusion NS1B protein to complex with the bound GST–NS1B sequence was determined (Fig. 4B). The two amino-terminal polypeptides, NS1B (1–103) and NS1B (1–93), which retained all the RNA-binding activities of the full-length NS1B protein, also retained all the activity for complexing with the full-length NS1B protein (Fig. 4B, lanes 2–4). In contrast, the two amino-terminal peptides that lacked RNA-binding activities, NS1B (1–78) and NS1B (1–68), possessed little or no activity for complexing with the full-length NS1B protein (lanes 5 and 6). Consequently, the RNA-binding domain was both necessary

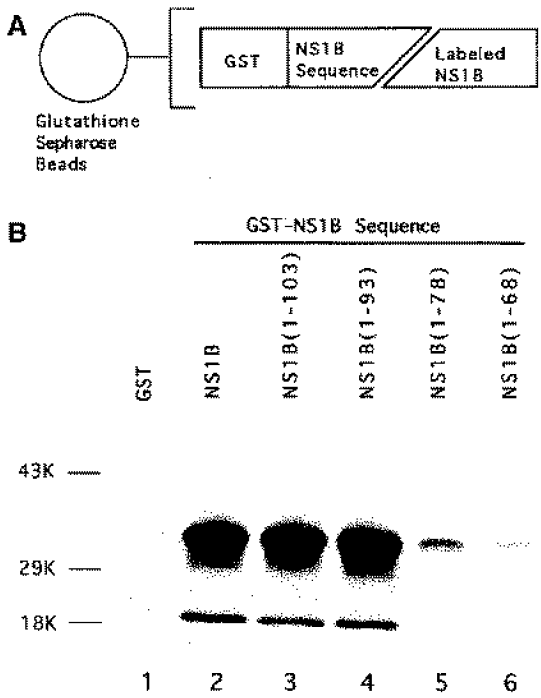

FIG. 4. The NS1B protein interacts with itself *in vitro* via its RNA-binding domain. (A) Schematic diagram of the glutathione Sepharose selection assay. (B) Binding of ^{35}S-labeled full-length NS1B protein to purified GST fusion protein containing either the entirety of the NS1B protein (lane 2) or various lengths (68, 78, 93, or 103 amino acids) of the amino terminus of the NS1B protein (lanes 3–6). Five microliters of the ^{35}S-labeled, full-length NS1B protein was incubated with 20 μl of glutathione Sepharose 4B beads and 2 μg of the GST fusion protein containing the indicated NS1B sequence. Lane 1, GST protein with no NS1B sequences. The ^{35}S-labeled, full-length NS1B protein that was bound to the beads was eluted and analyzed by electrophoresis on a 14% SDS–PAGE gel. Protein molecular weight markers are indicated.

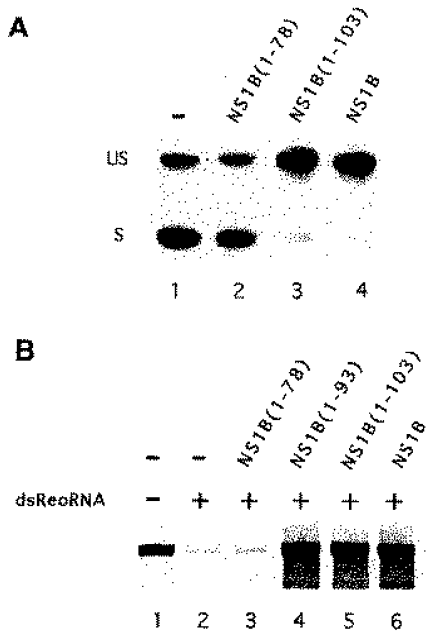

FIG. 5. The NS1B protein and its amino-terminal fragment inhibit splicing and block PKR activation *in vitro*. (A) Splicing. ^{32}P-labeled globin pre-mRNA was incubated for 2 hr at 30° with 10 μl HeLa cell nuclear extract under splicing conditions in the absence (–) (lane 1) or the presence of 1.0 μM of the indicated NS1B sequence (lanes 2–4). The RNA products were analyzed by gel electrophoresis. US, unspliced globin pre-mRNA; S, spliced globin mRNA. (B) PKR activation. Reovirus dsRNA (1 μg/ml) was incubated with 200 nM of the indicated NS1B sequence for 10 min at room temperature (lanes 3–6). As a control, no protein was added to the reovirus dsRNA (lane 2). The subsequent translation reaction was carried out as described under Materials and Methods. The amount of protein synthesized was determined by gel electrophoresis on a 14% SDS–PAGE gel followed by fluorography. Lane 1, no reovirus dsRNA was added to the reticulocyte extract.

and sufficient for complexing with the full-length NS1B protein. Gel filtration established that, as is the case for the NS1A protein (Nemeroff *et al.*, 1995; Qian *et al.*, 1995), the NS1B protein complex is in the form of a dimer (data not shown). Formation of NS1A–NS1B heterodimers was not detected (data not shown).

The NS1B protein and its amino-terminal fragment inhibit pre-mRNA splicing and block the activation of the PKR kinase *in vitro*

The binding of the NS1A protein to U6 snRNA molecules present in nuclear extracts causes the inhibition of pre-mRNA splicing catalyzed by these extracts *in vitro* (Lu *et al.*, 1995; Qiu *et al.*, 1995). As shown in Fig. 5A, the NS1B protein exhibited the same inhibitory activity. Thus, the addition of the NS1B protein (1.0 μM) caused inhibition of the splicing of a globin pre-mRNA (compare lanes 1 and 4). Splicing inhibition correlated with U6 snRNA-binding activity: an amino-terminal fragment of NS1B that possessed U6 snRNA-binding activity, NS1B (1–103), inhibited pre-mRNA splicing (lane 3), whereas an amino-terminal fragment that lacked U6 snRNA-binding activity, NS1B (1–78), did not inhibit pre-mRNA splicing (lane 2).

The addition of dsRNA to reticulocyte extracts activates the endogenous PKR kinase, thereby causing an inhibition of translation (Fig. 5B, compare lanes 1 and 2) (reviewed in Samuel, 1993). It was shown previously that the NS1A protein, by binding to dsRNA, blocks the activation of the PKR kinase and the resulting inhibition of translation *in vitro* (Lu *et al.*, 1995). The NS1B protein also blocked the inhibition of translation (lane 6), indicating that PKR activation by the added dsRNA was blocked. The block of translation inhibition correlated with dsRNA-binding activity: two amino-terminal fragments that possessed dsRNA-binding activity, NS1B (1–93) and NS1B (1–103), blocked the inhibition of translation (Fig. 5B, lanes 4 and 5), whereas an amino-terminal fragment that lacked dsRNA-binding activity, NS1B (1–78), did not block the inhibition of translation (lane 3).

The activities of the NS1A and NS1B proteins as inhibitors of pre-mRNA splicing and the nuclear export of mRNA *in vivo*

The above results indicate that the NS1A and NS1B proteins possess RNA-binding domains with similar activities. The NS1A protein possesses a second important domain, the effector domain, that is required for its two measurable *in vivo* activities, the inhibition of the nuclear export of poly(A)-containing mRNAs and the inhibition of pre-mRNA splicing (Lu *et al.*, 1994; Qian *et al.*, 1994; Qiu and Krug, 1994). To determine whether the NS1B protein possesses an effector domain like that of the NS1A protein, 293 cells were transfected with two plasmids: one specifying either the NS1A or the NS1B protein and one specifying either a globin minigene pre-mRNA to assay splicing (Fig. 6A) or a spliced globin mRNA to assay nuclear export of mRNA (Fig. 6B). The NS1A and NS1B proteins had very different effects on globin pre-mRNA splicing *in vivo* (Fig. 6A). In the presence of the NS1B protein efficient splicing of the globin pre-mRNA occurred: the ratio of unspliced to spliced globin mRNA was approximately 1:7 (Fig. 6A, lane 2). This is the same extent of splicing that occurred in the absence of the NS1B protein (Fig. 6A, lane 1). In contrast, in the presence of the NS1A protein splicing of the globin pre-mRNA was strongly inhibited: the ratio of unspliced to spliced globin mRNA was reversed, to a ratio of approximately 4:1 (Fig. 6A, lane 3), in confirmation of previous results (Lu *et al.*, 1994). The NS1A and NS1B proteins also had very different effects on the nuclear export of spliced globin mRNA (Fig. 6B). In the presence of the NS1B protein the majority (70%) of the spliced globin mRNA was transported to the cytoplasm (Fig. 6B, lanes 3 and 4), the same extent of

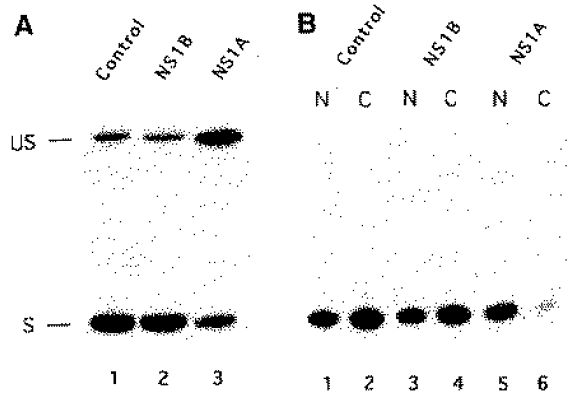

FIG. 6. The NS1A, but not the NS1B, protein inhibits pre-mRNA splicing and the nuclear export of poly(A)-containing mRNA *in vivo*. (A) Splicing *in vivo*. 293 cells were cotransfected with 5 μg of a pBC12 plasmid encoding globin pre-mRNA and 10 μg of a pBC12 plasmid encoding no protein (control, lane 1), the NS1B protein (lane 2), or the NS1A protein (lane 3). At 40 hr posttransfection, the cells were harvested. Total RNA was extracted and subjected to S1 analysis using a 5′-end-labeled, single-stranded DNA probe specific for globin pre-mRNA and spliced mRNA (Lu *et al.*, 1994). The protected fragments were resolved by denaturing gel electrophoresis, as shown. US, unspliced; S, spliced globin mRNAs. (B) Nuclear export of poly(A)-containing mRNA. The pBC12 plasmid (10 μg) encoding no protein (lanes 1 and 2), the NS1B protein (lanes 3 and 4), or the NS1A protein (lanes 5 and 6) was cotransfected with 5 μg of a pBC12 plasmid encoding spliced globin mRNA. At 40 hr posttransfection, the cells were fractionated into nuclei and cytoplasm. Cell equivalent amounts of nuclear (N) and cytoplasmic (C) RNAs, namely, 0.5 and 5 μg, respectively, were extracted and subjected to S1 analysis.

transport that occurred in the absence of the NS1B protein (lanes 1 and 2). In contrast, in the presence of the NS1A protein, most (95%) of the spliced globin mRNA was retained in the nucleus (lanes 5 and 6), in confirmation of previous results (Alonso-Caplen *et al.*, 1992; Qian *et al.*, 1994). We verified that, as reported previously (Norton *et al.*, 1987), the NS1B protein, like the NS1A protein, was localized in the nucleus and that similar amounts of the NS1A and NS1B proteins were synthesized in the transfected cells (data not shown).

These results indicate that the NS1B protein lacks an effector domain that functions like the NS1A protein effector domain *in vivo*. Consequently, the NS1B protein would be expected to behave like the NS1 protein of a naturally occurring influenza A virus strain, A/Turkey/Oregon/71, which is only 125 amino acids long (Norton *et al.*, 1987). This NS1A protein contains the amino half of the molecule where the RNA-binding domain is located, but lacks the carboxyl region where the effector domain normally located. This truncated NS1A (Turkey NS1A) protein binds to the same three RNA targets as the full-length NS1A and NS1B proteins (data not shown), and as a result of its binding to U6 snRNA inhibits pre-mRNA splicing *in vitro* (Fig. 7A). However, when the plasmid encoding the Turkey NS1A protein was transfected into 293 cells, the splicing of a globin pre-mRNA was not inhibited (Fig. 7B, compare lanes 1 and 2), nor was the nuclear export of globin spliced mRNA inhibited (data not shown). The full-length NS1A protein used as a control for this experiment is encoded by the A/Duck/Alberta/60/76 NS1 gene (Duck NS1A), which is 100% homologous with the A/Turkey/Oregon/71 NS1 gene except for the absence of the 10-nucleotide deletion that caused the truncation of the Turkey NS1A protein (Norton *et al.*, 1987). The Duck NS1A protein effectively inhibited globin pre-mRNA splicing *in vivo* (Fig. 7B, lane 3), as well as the nuclear export of globin spliced mRNA (data not shown). This indicates that the Duck NS1A protein, which has a sequence that significantly diverges (80% similarity, 68% identity) from that of the NS1 protein of A/Udorn/72 (Baez *et al.*, 1981; Ludwig *et al.*, 1991), possesses a functional effector domain.

DISCUSSION

Previous studies have shown both similarities and differences between the gene products of influenza A viruses and those of influenza B viruses (reviewed in Lamb, 1989). Consistent with this pattern, the present study demonstrates that the NS1 proteins of these two influenza virus types exhibit both similarities and differences.

One of the two functional domains of the NS1A protein, the RNA-binding domain, is preserved in the NS1B protein, even though there is little or no sequence homology between the two proteins (Briedis and Lamb, 1982). The

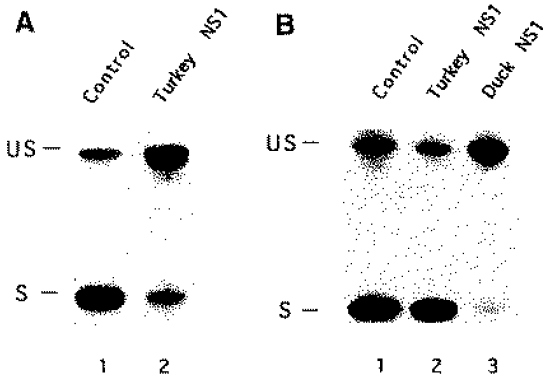

FIG. 7. The truncated NS1 protein of influenza virus A/Turkey/Oregon/71 (Turkey NS1) inhibits splicing *in vitro* but not *in vivo*. (A) *In vitro*. ^{32}P-labeled globin pre-mRNA was incubated for 2 hr at 30° with HeLa cell nuclear extract under splicing conditions in the absence (control) (lane 1) or presence (lane 2) of 1.5 μ*M* of the Turkey NS1 protein. The RNA products were analyzed by gel electrophoresis. US, unspliced; S, spliced. (B) *In vivo*. 293 cells were cotransfected with 5 μg of a pBC12 plasmid encoding globin pre-mRNA and 10 μg of a pBC12 plasmid encoding no protein (control, lane 1), the Turkey NS1 protein (lane 2), or the Duck NS1 protein (lane 3). At 40 hr posttransfection, the cells were harvested and the extracted RNAs were subjected to S1 analysis. The protected fragments were resolved by gel electrophoresis, as shown. US, unspliced; S, spliced globin mRNA.

NS1B protein specifically binds to the same RNA targets as the NS1A protein, namely, poly(A), a stem-bulge in U6 snRNA, and dsRNA (Qiu and Krug, 1994; Lu *et al.*, 1995; Qiu *et al.*, 1995; present study). For both the NS1A and NS1B proteins, the binding to U6 snRNA causes an inhibition of pre-mRNA splicing *in vitro*, and the binding to dsRNA blocks the activation of the PKR kinase *in vitro* (Lu *et al.*, 1994, 1995; present study). As is the case with the NS1A protein (Qian *et al.*, 1995), a polypeptide containing an amino-terminal sequence of the NS1B protein possesses all the RNA-binding activity of the full-length protein and exists in the form of a dimer. Structural studies, using both circular dichroism (CD) and nuclear magnetic resonance, indicate that the amino-terminal polypeptide of the NS1A protein is largely α-helical (Qian *et al.*, 1995); and CD analysis indicates that the amino-terminal polypeptide of the NS1B protein shares this property (unpublished data). Thus, though the RNA-binding domains of the NS1A and NS1B proteins have little or no sequence homology (Briedis and Lamb, 1982), they most likely share a structural homology. The attainment of this structural homology required amino acid sequences of different lengths: the minimum length of the amino-terminal NS1A polypeptide that possessed RNA-binding activity was 73 amino acids, whereas the corresponding NS1B polypeptide was 93 amino acids.

In contrast to the preservation of the RNA-binding domain, the second major functional domain in the NS1A protein, the effector domain, was most likely not preserved in the NS1B protein. The effector domain of the NS1A protein, which is located in the carboxyl half of the protein, is required for two of the *in vivo* activities of the NS1A protein, the inhibition of the nuclear export of mRNA and the inhibition of pre-mRNA splicing (Qian *et al.*, 1994; Lu *et al.*, 1995). As shown here, the NS1B protein lacks these *in vivo* activities. In cells transfected with the NS1B gene neither pre-mRNA splicing nor the nuclear export of mRNA was inhibited. Like the NS1B protein, the NS1 protein of a naturally occurring influenza virus A strain that contains a deletion of its effector domain region (Norton *et al.*, 1987) has the RNA-binding activity of the full-length NS1A protein, but fails to inhibit pre-mRNA splicing and the nuclear export of poly(A)-containing mRNA *in vivo*. These results strongly suggest that the full-length NS1B protein lacks an effector domain that functions like that of the full-length NS1A protein.

The conservation of the RNA-binding domain of the NS1 protein among influenza A and B viruses strongly suggests that this domain, which binds three RNA targets, poly(A), U6 snRNA, and dsRNA, is required for the replication of these two types of influenza viruses. In contrast, the lack of conservation of the effector domain among influenza virus A and B viruses argues that this domain is not absolutely required for the replication of these two types of influenza viruses. These conclusions raise several interrelated questions.

First, why would the RNA-binding domain of the NS1 protein be preserved in all influenza virus A and B viruses if the binding to two of its targets, poly(A) and U6 snRNA, serves no apparent purpose *in vivo* when the protein lacks a functional effector domain? The most plausible explanation is that the *in vivo* function mediated by the binding of the NS1 protein to dsRNA, the third RNA target, is absolutely required for virus replication. Influenza virus makes large amounts of both negative-strand (i.e., virion-sense) and positive-strand viral RNAs during infection, and as a consequence viral dsRNAs are formed that are capable of activating the cellular PKR kinase (Katze, 1992). This would cause an inhibition of the translation of both viral and cellular mRNAs. To block the activation of the PKR kinase, influenza virus apparently mounts a two-pronged attack. It has been shown that the virus activates a 58-kDa protein that inhibits the phosphorylation of the α subunit of eIF2 by the PKR kinase (Lee *et al.*, 1990; 1992). In addition, the viral NS1 protein would bind to and sequester dsRNA, thereby prevent the activation of the PKR kinase (Lu *et al.*, 1995). It can be postulated that this *in vivo* function of the NS1 protein does not require its effector domain, unlike the *in vivo* functions of this protein in pre-mRNA splicing and in the nuclear export of poly(A)-containing mRNA (Qian *et al.*, 1994; Lu *et al.*, 1995). Clearly, an important goal will be to determine whether the effector domain of the NS1 protein is required for its ability to block the activation of the PKR kinase *in vivo*. Efficient blocking of PKR kinase activation assumes even more importance during influenza virus infection of its vertebrate host, where virus infection induces the synthesis of interferon that in turn induces the synthesis of increased amounts of PKR kinase (reviewed in Katze, 1992; Rhoads, 1993; Samuel, 1993). According to this hypothesis, the essential function of the NS1 protein for all influenza virus A and B viruses would be to protect against the activation of the PKR kinase and hence against the resulting inhibition of translation that would block virus replication.

Second, we have shown that a functional effector domain is present in two full-length NS1A proteins that exhibit one of the widest divergences in NS1 sequence among influenza A viruses, those encoded by the A/Udorn/72 and A/Duck/Alberta/60/76 viruses. We can conclude that a functional effector domain is most likely preserved in all full-length NS1A proteins. Why is this effector domain preserved in all these NS1A proteins, in light of the demonstration that an effector domain like that of the NS1A protein is not required for the replication of influenza B viruses nor for the replication of at least one influenza A virus? It has been proposed that, as a consequence of the presence of a functional effector domain, NS1A proteins would be able to serve several

additional functions in infected cells (Lu *et al.*, 1994; Qiu and Krug, 1994). Because of their ability to inhibit pre-mRNA splicing and the nuclear export of poly(A)-containing mRNA, these NS1A proteins would be able to sequester cellular pre-mRNAs and mRNAs in the nucleus. Consequently, more of these cellular capped RNAs would be accessible to the viral cap-dependent endonuclease in the nucleus for the production of the capped RNA primers that are needed for viral mRNA synthesis (reviewed in Krug *et al.*, 1989). This would also explain the observed degradation of cellular pre-mRNAs and mRNAs in the nucleus of infected cells (Katze and Krug, 1984). In addition, these NS1A proteins would be capable of regulating the early-to-late switch in viral protein synthesis by retaining the late viral mRNAs in the nucleus until the appropriate time for their expression. Is there some reason that almost all influenza A viruses retain the ability to carry out these functions, whereas influenza B viruses do not?

Finally, the NS1 proteins of almost all influenza B viruses possess a large carboxyl region that not functions like the effector domain of NS1A protein. What is the function of the carboxyl region of these NS1B proteins, particularly in light of the existence of a laboratory B virus variant with a truncated NS1 protein lacking the carboxyl region (Norton *et al.*, 1987)? The answers to these questions should provide insights into the different biological properties of influenza A and B viruses.

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